Neuroprotective Effect of Acute Ethanol Administration in a Rat With Transient Cerebral Ischemia

Fei Wang, MD*; Yu Wang, MD*; Xiaokun Geng, MD; Karam Asmaro, MS; Changya Peng, MS; Jonathon M. Sullivan, MD, PhD; Jamie Y. Ding, BS; Xunming Ji, MD, PhD; Yuchuan Ding, MD, PhD

Background and Purpose—Ethanol consumption is inversely associated with the risk of ischemic stroke, suggesting a neuroprotective effect. In a rat model of transient cerebral ischemia, we identified ethanol as a possible treatment for acute ischemic stroke.

Methods—Sprague-Dawley rats were subjected to middle cerebral artery occlusion for 2 hours. Five sets of experiments were conducted: to determine the dose–response effect of ethanol on brain infarction and functional outcome; to determine whether combining ethanol and hypothermia produces synergistic neuroprotection; to determine the therapeutic windows of opportunity for ethanol in stroke; to test whether ethanol promotes intracerebral hemorrhage in a hemorrhagic or ischemic stroke or after administration of thrombolytics; and to test the affect of ethanol on hypoxia-inducible factor-1α protein expression.

Results—Ethanol at 1.5 g/kg reduced infarct volume and behavioral dysfunction when administered at 2, 3, or 4 hours after middle cerebral artery occlusion. The protective effect of ethanol was not improved when paired with hypothermia. Ethanol did not promote cerebral hemorrhage in hemorrhagic or ischemic stroke in combination with recombinant tissue-type plasminogen activator or urokinase. Ethanol treatment (1.5 g/kg) increased protein levels of hypoxia-inducible factor-1α at 3 hours postreperfusion.

Conclusions—Ethanol exerts a strong neuroprotective effect when administered up to 4 hours after ischemia, increases expression of hypoxia-inducible factor-1α, and does not promote intracerebral hemorrhage when used with thrombolytics. Ethanol is a potential neuroprotectant for acute ischemic stroke. (Stroke. 2012;43:205-210.)

Key Words: cerebral ischemia ■ ethanol ■ neuroprotection

The lack of clinically effective neuroprotectants for stroke demands efforts directed at the establishment of new approaches to therapy. Despite a focus on the pathological effects of alcohol abuse, numerous studies published over the past several decades also demonstrate the beneficial effects of light-to-moderate consumption of alcoholic beverages. Elevated serum ethanol levels are independently associated with increased survival in patients with traumatic brain injury, suggesting that acute exposure to alcohol exerts a neuroprotective effect. Nevertheless, the potential neuroprotective benefits of acute alcohol intoxication in ischemic stroke remain unclear. For example, Phillis et al demonstrated that an acute ethanol dose of 22 mmol/kg (1.02 g/kg) reduced neuronal death in a gerbil global ischemia model, whereas Crews et al showed a neuroprotective effect of ethanol in doses of 3 g/kg was abolished under normothermic conditions during ischemia.

In this report, we demonstrate dose–response and therapeutic windows for ethanol in acute ischemic stroke as well as interactions with hypothermia and thrombolytics.

To understand the mechanisms underlying ethanol-induced neuroprotection, we also determined whether poststroke ethanol administration upregulates hypoxia-inducible factor (HIF)-1α, which has been reported to likely mediate adaptive functions by regulating multiple genes.

Materials and Methods

All experiments were approved by the Institutional Animal Investigation Committee of our institutions and were in accordance with National Institutes of Health guidelines for care and use of laboratory animals. A total of 160 adult (280–300 g) male Sprague-Dawley rats (Charles River, Wilmington, MA; and Vital River Laboratory Animal Technology Co Ltd, Beijing, China) were used. Rats were randomly divided into a sham-operated group and 4 stroke groups (n=8), including 1 nontreatment group and 3 treatment groups, with...
each treatment group randomly receiving a different dose (0.5, 0.1, or 1.5 g/kg) of ethanol after 2 hours of middle cerebral artery occlusion (MCAO) followed by 3-, 24-, or 48-hour reperfusion. We investigated the therapeutic window in ischemic rats by administering 1.5 g/kg of ethanol either immediately before or during reperfusion: 2, 3, or 4 hours after MCAO. Collagenase injections were used to induce intracerebral hemorrhage (ICH) for hemorrhagic stroke evaluation. Finally, we determined protein expression of HIF-1α poststroke ethanol administration. Mortality rate was approximately equal from each group (14% to 18%). All data were analyzed in a blinded manner.

Focal Cerebral Ischemia
MCAO was induced using the intraluminal filament model described by Zea Longa et al.6 Blood pCO2, and pO2, mean arterial pressure as well as rectal and brain temperature were monitored during the procedure. The rectal temperature was maintained at approximately 36.5°C to 37.5°C using a circulating heating pad and a heating lamp.

Ethanol Treatment
Rats in the stroke groups were administered either ethanol (0.5, 1.0, or 1.5 g/kg) diluted to 3.0 mL in normal saline or normal saline (3.0 mL) by intraperitoneal injection either before reperfusion or at specified time points after reperfusion (Figure 1A). Ethanol at dose of 1.5 g/kg was administered 2 hours after ICH induction. Animals were euthanized at 3, 24, or 48 hours after reperfusion or 3 hours after ICH for histological or molecular analyses or 28 days after reperfusion for behavior tests.

Ethanol and Hypothermia
To determine whether ethanol and hypothermia produced synergistic neuroprotection, we induced 32°C to 33°C body core hypothermia by spraying 75% alcohol onto the entire body of the rat.7 Contact with exposed skin (as opposed to fur) is minimal, and there is no evidence of clinically relevant transdermal absorption.8 Hypothermia was induced 90 minutes after MCAO with and without ethanol treatment (1.5 g/kg administered 2 hours after MCAO) and was sustained until the onset of reperfusion followed by spontaneous rewarming.

Recombinant Tissue-Type Plasminogen Activator Administration
To determine the effect of ethanol on recombinant tissue-type plasminogen activator (rtPA)-induced hemorrhage, ethanol (1.5 g/kg) was administrated by an intraperitoneal injection immediately before the onset of reperfusion; and a total dose of 5 mg/kg (2.5-mg/kg bolus followed by 30-minute infusion at 5 mg/kg per hour) of rtPA (Actilyse 50 mg/ Vial; Boehringer Ingelheim Pharma) was administered intravenously 15 minutes after the onset of reperfusion.9

Urokinase Administration
To determine the effect of ethanol on urokinase (UK)-induced hemorrhage, ethanol (1.5 g/kg) was administrated to ischemic rats undergoing UK treatment. A total of 170,000 IU/kg UK (500 000 IU/vial; Livzon Pharmaceutical Group Inc) dissolved in saline to a volume of 1.5 mL10 was injected into the middle cerebral artery territory through an internal carotid catheter using a tetherless infusion pump (Pegasus) set at a speed of 4000 IU/min.

Evaluation of Cerebral Infarct Volume
Forty-eight hours after reperfusion, brains were harvested and cut into 2-mm thick coronal slices for 2,3,5-triphenyltetrazolium chloride (Sigma) treatment.8 Brain slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride solution at 37°C for 20 minutes. Stained slices were fixed in 10% formalin solution. To minimize error introduced by edema, an indirect method for calculating infarct volume was used.

Figure 1. Ethanol minimizes ischemic brain injury in the rat focal ischemic stroke model. A, Representative scheme showing the addition of ethanol at the various indicated time points for the studies illustrated. B, Representative brain sections 48 hours after reperfusion. C, Quantitative analysis of infarct volume in each of the 4 study groups (n=8 per group). Ethanol significantly minimizes infarct area at concentrations of 1.0 g/kg (**P<0.01) and 1.5 g/kg (**P<0.001) when compared with controls treated with saline.

Motor Behavioral Testing
Rats were examined using 3 different motor testing paradigms (foot fault placing, parallel bar crossing, and beam balance test) at 2, 4, 7, 10, 14, and 28 days after the surgical procedure.11

Intracerebral Hemorrhage
To further determine the effect of ethanol on brain hemorrhage, we administered ethanol in a collagenase-induced hemorrhagic model as previously described.12 Collagenase (0.5 U VII-S; Sigma) dissolved in 1 µL of saline was injected into the right striatum through a 22-gauge needle over 5 minutes. Cerebral hemorrhage was quantified by spectrophotometric assay.13

Protein Expression of HIF-1α
Western immunoblot analysis was used to assess protein expression of HIF-1α. Brain tissue samples containing frontoparietal cortex and dorsolateral striatum were processed as described previously14 using primary polyclonal rabbit anti-HIF-1α (1:2000; Santa Cruz).
Statistical Analysis
All values are expressed as means±SE. Statistical significance was determined either by analysis of variance supplemented by post hoc test (Duncan multiple range) for comparison of >2 groups or by Student t test for comparison between 2 groups. Statistical significance was accepted at P<0.05. We used SPSS software, Version 17.

Results

Physiological Parameters
There were no significant differences in blood pH, pO2, and pCO2 in ischemic rats with or without ethanol treatment (Supplemental Table I; http://stroke.ahajournals.org). However, a slight but significant (P<0.05) increase in arterial blood pressure was found in the ethanol-treated groups. Body and brain temperature remained at approximately 37°C.

Infarct Volume and Behavioral Function
After MCAO for 2 hours and reperfusion for 48 hours, we observed a dose-dependent correlation between ethanol dose (0.5, 1.0, and 1.5 g/kg) and infarct volume in stroke (F[3, 28]=39.7; P<0.001). Treatment with 1.5 g/kg of ethanol (28.4%±2.0%) was most effective, reducing total infarct volume by 47% compared with ischemic rats with sham treatment (53.8%±2.0%; Figure 1C). In contrast, 0.5 g/kg ethanol did not reduce brain injury (50.0%±1.9%). A smaller (23%) but significant (P<0.01) reduction in infarct volume (41.7%±1.1%) was seen with 1.0 g/kg.

Functional deficits were examined in 3 separate groups (control, stroke, and stroke with 1.5 g/kg ethanol) 2, 4, 7, 10, 14, and 28 days after ischemia/reperfusion using 3 motor tasks (Figure 2). Statistically significant results were detected among all 3 groups: for forelimb foot fault placing (Figure 2A; F[2, 22]=6.1, P<0.01), parallel bar traversing (Figure 2B; F[2, 23]=14.8, P<0.001), and beam balance (Figure 2C; F[2, 22]=22.7, P<0.001). The post hoc test indicated significant improvements in motor behavior 2 to 28 days after stroke in rats with ethanol treatment versus those without ethanol treatment, suggesting that acute ethanol improves functional outcome in stroke.

Ethanol in Combination With Hypothermia
The neuroprotective effects of ethanol (1.5 g/kg; 2 hours after stroke onset), hypothermia, and ethanol combined with hypothermia were compared by determining infarct volume (Figure 3A) and functional deficit (Figure 3B-D) for each treatment group. Compared with the control treatment group (saline), infarct volumes were significantly (P<0.05) reduced by ethanol (28.4%±2.0%), hypothermia (29.1%±1.2%), and ethanol and hypothermia combined (26.6%±2.9%). However, there were no statistically significant differences among the 3 treated groups; ethanol and hypothermia treatments together did not exert a synergistic effect on infarct volume.

Animals from the 4 groups were evaluated on days 2, 4, 7, 10, 14, and 28 post-MCAO and reperfusion based on 3 motor tasks. Statistically significant effects were detected among all 3 groups when compared with the control: for forelimb foot fault placing (Figure 3B; F[2, 23]=6.08, P<0.01), parallel bar traversing (Figure 3C; F[2, 23]=14.75, P<0.01), and beam balance (Figure 3D; F[2, 23]=22.70, P<0.01). The post hoc test indicated that significant improvements in motor behavior were achieved by each treatment in ischemic rats from 2 through 28 days after stroke. However, like with infarct volume, there were no significant differences found among the treatment groups. Again, combined hypothermia and ethanol treatment did not exert a synergistic effect on neurobehavioral outcome in this study.

Ethanol Administered at Various Time Points

After Reperfusion
The neuroprotective effects of ethanol at a dose of 1.5 g/kg when administered 2, 3, or 4 hours after MCAO were quantified by measuring infarct volume after 2-hour MCAO and 48-hour reperfusion. We found a significant (F[3, 28]=39.7, P<0.001) reduction in infarct volume when ethanol (1.5 g/kg) was administered up to 4 hours after MCAO, suggesting a therapeutic window for this therapy (Figure 4). Although infarct volume in rats subjected to ethanol treatment 4 hours after MCAO is greater than that at 2 or 3 hours, the post hoc test indicated that brain damage
Effects of Ethanol Administration on ICH in Hemorrhagic or Ischemic Stroke After Thrombolytic Therapy

Ethanol administration (1.5 g/kg; 2 hours after stroke onset) did not significantly increase cerebral bleeding after ischemic stroke (Figure 5A). In the collagenase ICH stroke model (Figure 5B), striatal hematomas were not enlarged by ethanol administration. We did not detect a significant difference ($P > 0.89$) between bleeding in the saline/rtPA-treated group (9.80 ± 1.80 μL) and that in the ethanol/rtPA-treated group (10.12 ± 1.55 μL; Figure 5C). Bleeding in rats subjected to ischemia/reperfusion with saline/UK (10.90 ± 1.38 μL) was not significantly different ($P > 0.73$) from that of rats subjected to ischemia/reperfusion with ethanol/UK (11.69 ± 1.81 μL; Figure 5D).

Protein Expression of HIF-1α

A total of 1.5 g/kg ethanol significantly ($F_{1,2,15} = 213.2$, $P < 0.01$) increased the expression of HIF-1α by 63% at 3 hours after reperfusion when compared with the control group (Figure 6). Although a significant difference ($F_{1,2,15} = 7.6$, $P < 0.001$) was found among groups at 24 hours postreperfusion, no significant change in HIF-1α expression was detected by ethanol administration.

Discussion

We observed a neuroprotective effect of 1.5 g/kg of ethanol when administered within 4 hours after the onset of MCAO. We find that ethanol and moderate hypothermia (32–34°C) exert neuroprotective effects of similar magnitude at both the lesion volume and functional levels but do not exert a synergistic effect in this model. Ethanol does not enhance brain hemorrhage in ischemic animals, in collagenase-induced ICH, or after the administration of the thrombolytics rtPA and UK. Upregulation of HIF-1α protein levels was enhanced by ethanol in association with reduced brain infarct volume and improved functional recovery in rats subjected to stroke using the same dose.
Alcohol has documented therapeutic potential for traumatic brain injury. Tien et al first reported significant differences in mortality in patients with traumatic brain injury with different serum alcohol levels.\textsuperscript{15} The low-to-moderate alcohol group (\(0.230\) mg/dL) exhibited better survival than the no-alcohol group (0 mg/dL). In contrast, compared with the same no-alcohol group, the high alcohol group (\(2.30\) mg/dL) demonstrated worse survival rates. In experimental focal cerebral ischemia in rats,\textsuperscript{16} acute ethanol effects also indicated a significant dose–effect relationship in which the lower dose of ethanol (2 g/kg) reduced ischemic core water content, and the larger dose of 3 g/kg increased brain edema and produced a mean whole blood level of ethanol at 230 mg/dL. In other studies, low-dose ethanol (0.2–0.65 g/kg, producing blood levels of <46 mg/dL) demonstrated no neuroprotection after stroke if ethanol was not combined with caffeine.\textsuperscript{9,17} Our study suggests that ethanol doses of 1.0 to 1.5 g/kg, which produce blood levels (\(89\) mg/dL)\textsuperscript{18} within the legally intoxicated range (80–100 g/dL),\textsuperscript{15} are suitable for study of neuroprotective effects and mechanisms.

For the present study, we hypothesized that the neuroprotective effect of ethanol could be amplified by combining it with therapeutic hypothermia, because hypothermia has a strong neuroprotective effect in cardiac arrest and cerebral ischemia.\textsuperscript{19} However, our data do not support a synergistic effect of hypothermia and ethanol when used as combined therapy. We demonstrated comparable neuroprotection with both treatments, suggesting that hypothermia and ethanol may share a similar mechanism. In this regard, the neuroprotective mechanism appears to be attributable in part to reduced cerebral metabolism.\textsuperscript{18,20,21} Previous studies on traumatic brain injury have indicated an increase in cerebral blood flow after ethanol administration using similar dosage (1.0 g/kg).\textsuperscript{18} The authors demonstrated that cerebral blood flow in both contusion core and penumbra were elevated in the ethanol treatment group up to 72 hours.

It has been suggested that ethanol may produce vascular damage and hemorrhage,\textsuperscript{9} which would have implications for clinical use of this drug. In vitro studies show that the rat fibrinolytic system is 10-fold less sensitive to rtPA than the human system. Haelewyn et al showed that the dose of 0.9 mg/kg rtPA (the clinical dose in patients with stroke) is as appropriate as that of 10 mg/kg for preclinical stroke studies in rodents.\textsuperscript{22} Aronowski et al found that 10-mg/kg dose of intravenous rtPA started 5 minutes after 180 minutes of MCAO was very toxic and resulted in severe bleeding and high mortality in animals and suggested that 5 mg/kg rtPA produced bleeding in approximately 85% to 90% of animals.\textsuperscript{9}

Accordingly, we used a total dose of 5 mg/kg (2.5 mg/kg

**Figure 5.** Administration of 1.5 g/kg ethanol, 2 hours poststroke onset, does not induce cerebral bleeding. The amount of blood leakage in saline- and ethanol-treated ischemic (A) or ICH (B) rats (n=8 per group) was quantified. No significant difference was detected between the ischemic stroke group treated with saline vs ethanol. Ethanol did not enhance hemorrhage in conjunction with rtPA (C) or UK (D; n=8 per group). ICH indicates intracerebral hemorrhage; rtPA, recombinant tissue-type plasminogen activator; UK, urokinase.

**Figure 6.** Administration of 1.5 g/kg ethanol, 2 hours poststroke onset, significantly \((P<0.01)\) increases expression of HIF-1\(\alpha\) at 3 hours postreperfusion (n=8 per group). No further increase is seen after 24 hours with ethanol treatment. All values are a percent of the basal HIF-1\(\alpha\) protein levels in sham-operated animals (set at 1.0). Representative immunoblots are presented. Values are means±SE. HIF-1\(\alpha\) indicates hypoxia-inducible factor-1\(\alpha\).
bolus followed by 30-minute intravenous infusion at 5 mg/kg per hour) and rtPA was started 15 minutes after the onset of reperfusion. Our previous study showed that intra-arterial administration of UK at a dose of 170 000 IU/kg (infusion at 4000 IU/min) induced neuroprotection in the rat MCAO stroke model. In the present study, we observed that a neuroprotective dose of ethanol (1.5 g/kg) did not promote hemorrhage with or without thrombolytic therapy in the rat.

Previous studies, including ours, have suggested that 2-hour MCAO in rats leads to consistent and extensive cerebral infarction. We chose this time period for ischemia and extended therapeutic windows during reperfusion, targeting reperfusion injury. It is also important to look at how ischemia time (>2 hours) affects therapeutic windows in our future studies.

Our results implicate the expression of HIF-1α, a key regulator of oxygen homeostasis, as a potential mediator of ethanol-induced neuroprotection. HIF-1α regulates genes involved in glycolysis, erythropoiesis, angiogenesis, and apoptosis. Because its many target genes mediate both adaptive and pathological processes, the role of HIF-1α in neuronal survival is debated. Further studies are necessary to investigate the potential role of HIF-1α in ethanol-induced neuroprotection.

Sources of Funding
This work was partially supported by the American Heart Association and Neurosurgery Fund, National Natural Science Foundation of China (30870854), and National Basic Research Program of China (973 Program; 2011CB707804).

Disclosures
None.

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Stroke. 2012;43:205-210; originally published online November 3, 2011;
doi: 10.1161/STROKEAHA.111.629576

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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SUPPLEMENTARY DATA

Protein expression of hypoxia-inducible factor-1α (additional info) — Western immunoblot analysis was used to assess protein expression of HIF-1α. Brain tissue samples were processed as described previously, using primary polyclonal rabbit anti-HIF-1α (1:2,000, Santa Cruz). Equal protein loading was confirmed and adjusted by intracellular protein β-actin (goat polyclonal anti-β-actin antibody, 1:1,000, Santa Cruz). Targeted antigens were visualized using standard chemical luminescence methods (Amersham ECL, GE Healthcare BioSciences, Piscataway, NJ, USA). The relative levels of target protein expression were quantified using the program ImageJ 1.42 (NIH).

Focal cerebral ischemia (additional info) — Rats were fasted overnight, but were allowed free access to water. Blood pCO2, and pO2, blood pressure, as well as rectal and brain temperature were monitored during the procedure. The rectal temperature was maintained at about 36.5-37.5 °C using a circulating heating pad and a heating lamp. Animals were anesthetized with 1.5-2% enflurane in a 7:3 mixture of N2O and O2. Briefly, a filament (4–0 nylon suture with blunted tip) coated with poly-L-lysine was inserted into the right external carotid artery and lodged in the narrow proximal anterior cerebral artery, blocking the MCA at its origin. Two hours after occlusion, reperfusion was established by filament withdrawal. To verify MCAO, rats underwent neurological examination during the periods of both ischemia and reperfusion.

Motor behavioral testing (additional info) — Rats were examined using three different motor testing paradigms (foot fault placing, parallel bar crossing, and beam balance test) at 2, 4, 7, 10, 14, and 28 days after the surgical procedure. All rats were tested three times on each trial day; behavior analyses were performed blindly. A modified forelimb foot fault placing test was used to examine forelimb function. The foot-placing apparatus consisted of an elevated (100 cm) grid surface (106 × 110 cm, with a square opening of 9 cm² and grid wire diameter of 1.0 mm) connected to platforms at each end. Rats were stimulated to move across the grid surface either by noise or by prodding, and were observed for one minute. Occasionally, the rats placed a forelimb inappropriately, resulting in their fall through one of the openings in the grid. Such mistakes were counted as foot faults. The number of contralateral forelimb
foot faults made per meter in 1 minute was also calculated. Parallel bar testing was used to test hind limb coordination; this apparatus consisted of two parallel wooden rods (each 1.0 cm in diameter and 115 cm in length, with an inter-rod distance of 2.5 cm), connected to a platform at each end. The number of times the rat placed two hind paws on one rod, dropped a hind paw below the rod, fell, or swung under the rods in one minute was recorded. The balance beam test was utilized to assess for coordination and balance. Basically, animals were placed on wooden beams and were monitored for the duration (up to 60 seconds) they could remain on the beam.

RESULTS

There were no significant difference in blood pH, pO$_2$ and pCO$_2$ in ischemic rats with or without ethanol treatment. However, a slightly but significantly (P<0.05) increased arterial blood pressure was found ethanol-treated groups (Table 1).

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<th>TABLE 1: Physical Variables in Rats with Transient Ischemia and Reperfusion</th>
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*AVOVA indicates P<0.05 as compared to saline group; Recordings were collected after 1 hour of treatment; MAP: mean arterial pressure.
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   2000;20:396-404